Biochem. J. (1989) 261, 293–296 (Printed in Great Britain)

Synthesis of nitric oxide from L-arginine by neutrophils

Release and interaction with superoxide anion

Therese B. McCALL, Nigel K. BOUGHTON-SMITH, Richard M. J. PALMER, Brendan J. R. WHITTLE and Salvador MONCADA*

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

Stimulated rat peritoneal neutrophils release a platelet inhibitory factor with the pharmacological properties of NO. This release is inhibited by N^G-monomethyl-L-arginine and L-canavanine, indicating that it occurs through a mechanism similar to that in vascular endothelial cells and macrophages. As the degree of stimulation increases, the factor released is progressively inactivated by concomitant release of superoxide anions.

INTRODUCTION

Neutrophils (PMN) release a factor, with the pharmacological profile of NO, which relaxes vascular smooth muscle [1] and inhibits platelet aggregation [2]. When stimulated with a variety of substances, such as fMet-Leu-Phe (FMLP), PMN also release other factors, including superoxide anion (O_2^-; for a review, see [3]) which inactivates NO [4].

Vascular endothelial cells [5] and macrophages [6] synthesize NO from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine. This synthesis of NO is inhibited by N^G-monomethyl-L-arginine (L-NMMA), which also inhibits both endothelium-dependent vasodilatation *in vitro* and *in vivo* and some of the cytotoxic actions of macrophages (for a review see [7]).

We have now investigated the release from rat PMN of a platelet inhibitory factor with the properties of NO, its interaction with O_2^-, and the biochemical mechanism leading to its synthesis.

MATERIALS AND METHODS

Preparation of platelets and PMN

Human platelets, washed with prostacyclin, were prepared from citrated blood as described previously [8] and were resuspended in Tyrode's solution at a final concentration of 2×10^8 platelets·ml^{-1}.

Peritoneal PMN were elicited with oyster glycogen and harvested from male Wistar rats (200–250 g body wt.) as described previously [9]. The PMN were then purified by gradient centrifugation (250 *g* for 30 min) over Ficoll/Hypaque at room temperature. After hypo-osmotic lysis of erythrocytes, the PMN were resuspended at a final concentration of 10^7 cells·ml^{-1} in Tyrode's solution containing 1 mM-Ca^{2+} and 5 μM-indomethacin. The final cell preparation contained more than 95% PMN and was more than 98% viable as assessed by the uptake of Acridine Orange.

Platelet aggregation bioassay

Platelet suspensions (500 μl) were treated with indomethacin (5 μM) in a Payton aggregometer (37 °C, 900 rev./min) and aliquots (10–100 μl) of PMN added 4 min before addition of a submaximal concentration of thrombin (20–40 munits·ml^{-1}). Platelet aggregation was then monitored for 10 min. The anti-aggregating effect of PMN was expressed as a percentage (mean ± S.E.M.) of the aggregation (measured as increase in light transmission after 10 min) induced by thrombin alone. Student's t test (two-tailed) for unpaired data was used to determine statistical significance, and $P < 0.05$ was taken as statistically significant.

Determination of O_2^-

O_2^- generation by PMN was determined as reduction of cytochrome *c* [10], and the results were expressed as nmol of cytochrome *c* reduced in 2 min by 10^7 PMN.

Materials

Oyster glycogen, indomethacin, L-canavanine, superoxide dismutase (SOD) and cytochrome *c* (Sigma), human thrombin (Ortho Diagnostic Systems), Ficoll/Hypaque (Pharmacia), leukotriene B_4 (LTB_4; Professor E. J. Corey, Harvard University) and M&B 22948 (May and Baker) were obtained as indicated. Purified human haemoglobin [11] and L-NMMA and D-NMMA [12] were prepared as previously described.

RESULTS

Inhibition of platelet aggregation by PMN

PMN (10^5–10^6 cells) significantly inhibited platelet aggregation by 22±7–95±4% ($n = 8$) respectively. Treatment of PMN with 0.05% glutaraldehyde for 5 min reduced their viability to less than 10% and also reduced the inhibition induced by 10^6 cells from 91±5% to 10±4% ($n = 3$). The inhibitory activity of 3×10^5 cells (49±11%, $n = 10$) was significantly potentiated by 60

Abbreviations used: PMN, (polymorphonuclear) neutrophils; FMLP, fMet-Leu-Phe; O_2^-, superoxide anion; SOD, superoxide dismutase; LTB_4, leukotriene B_4; NMMA, *N*-monomethylarginine.

* To whom correspondence and reprint requests should be sent.

units of SOD·ml^{-1} to 88±7% (n = 8) or by 3 μM-M&B 22948 to 71±8% (n = 4) and was reduced to 20±9% (n = 3) by 200 nM-haemoglobin added 1 min before the addition of PMN.

Effect of L-arginine analogues

The inhibition of platelet aggregation by 3 × 10^5 PMN, in the presence of SOD, was reduced in a concentration-dependent manner by L-NMMA (30–300 μM), but not by D-NMMA (100 μM; Fig. 1*a*). The effect of L-NMMA was dependent on the time of preincubation with PMN, being maximal with a 50 min preincubation (n = 3). The maximum reduction observed was 53±9% at 300 μM-L-NMMA. The inhibition of anti-aggregating activity by L-NMMA (100 μM) was reversed by concomitant incubation with L-arginine, L-homoarginine, L-arginyl-L-aspartate and L-arginine methyl ester (Fig. 1*b*), but not by D-arginine (Fig. 1*b*) or L-citrulline (all at 300 μM; n = 3).

The anti-aggregating activity of 3 × 10^5 PMN in the presence of SOD (82±9%) was reduced to 71±7, 59±6 and 39±8% by 0.1, 0.3 and 1 mM-L-canavanine respectively (n = 3). The effect of L-canavanine (1 mM) was similarly dependent on the time of preincubation, being maximal at 50 min (n = 3) and was abolished by L-arginine (1 mM; n = 3). None of these concentrations of L-canavanine affected PMN viability (n = 3).

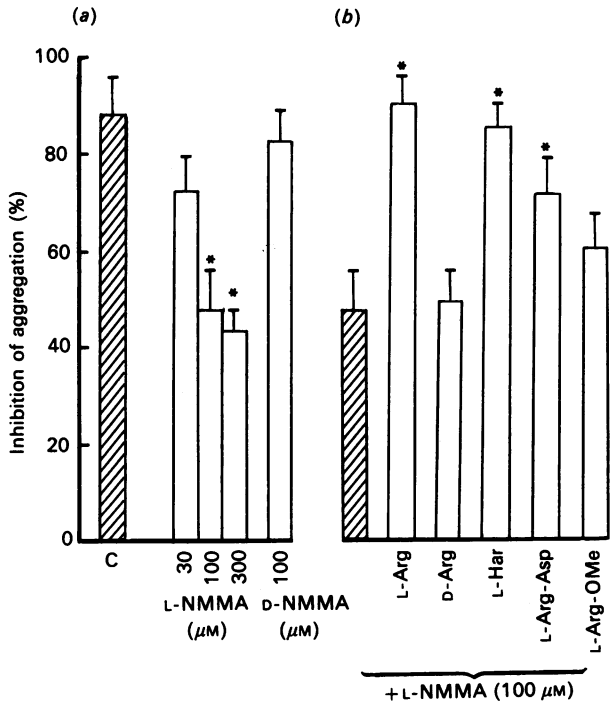

Fig. 1. (*a*) Inhibition by L-NMMA, but not D-NMMA, of the anti-aggregating activity of 3 × 10^5 PMN in the presence of SOD, and (*b*) reversal of the inhibition by L-NMMA (100 μM) of the anti-aggregating activity of PMN by L-arginine (L-Arg), L-homoarginine (L-Har), L-arginyl-L-aspartate (L-Arg-Asp) and L-arginine methyl ester (L-Arg-OMe), but not by D-arginine (D-Arg)

* Denotes significant difference from the corresponding control (hatched columns; n = 3).

Effect of FMLP and LTB_4

The inhibition of platelet aggregation by 3 × 10^5 PMN was significantly enhanced by low concentrations of FMLP, added to the platelets 1 min before the addition of PMN. Higher concentrations of FMLP, which did not affect platelet aggregation directly, reversed this effect and reduced the inhibition to below control levels (Fig. 2). The release of O_2^- induced by higher concentrations of FMLP was detectable using the cytochrome *c* assay (Fig. 2). The enhanced inhibition of platelet aggregation by 10^{-10} M-FMLP-stimulated PMN was reduced by 90±8% by haemoglobin (200 nM; n = 3) and by 88±6% by L-NMMA (100 μM; n = 3).

The effect of FMLP in the presence of SOD was investigated using 1 × 10^5 PMN with 60 units of SOD·ml^{-1}, which were as effective as 3 × 10^5 PMN alone in inhibiting platelet aggregation. Under these conditions, the enhancement of anti-aggregating activity of FMLP was significantly potentiated, and the reversal of this effect to below control levels by higher concentrations of FMLP was not observed (Fig. 2).

The inhibition of platelet aggregation by PMN was also significantly enhanced by LTB_4 (10^{-6} M; Fig. 3). The enhancement by LTB_4 of the anti-aggregating activity was potentiated by SOD (Fig. 3). The release of O_2^-, detected by reduction of cytochrome *c*, was only significant at the highest concentration of LTB_4 (Fig. 3).

L-Arginine (300 μM) alone did not affect the anti-aggregating activity of PMN. It did, however, potentiate significantly (from 69±5 to 93±4%) the enhancement of anti-aggregating activity of 3 × 10^5 PMN by a low concentration of FMLP (10^{-10} M) and prevented the reversal of this effect observed with a higher concentration (10^{-6} M) of the agonist (n = 3).

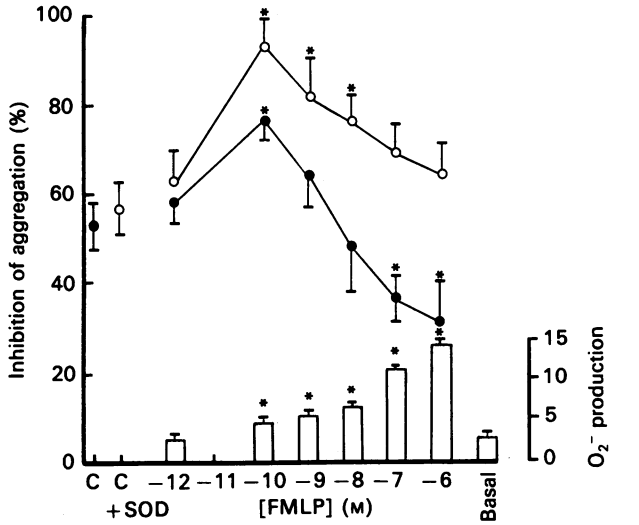

Fig. 2. Effect of FMLP on the inhibition of platelet aggregation and on O_2^- generation by PMN

'C' represents the anti-aggregating activity of 3 × 10^5 PMN alone and ● represents that after incubation with FMLP. 'C+SOD' represents the anti-aggregating activity of 1 × 10^5 PMN in the presence of SOD (60 units·ml^{-1}) alone, and ○ represents that after addition of FMLP. Basal and FMLP-stimulated O_2^- generation, expressed as nmol of cytochrome *c* reduced in 2 min by 10^7 cells, is shown by the histogram. * Denotes significant difference from the corresponding control (n = 4)

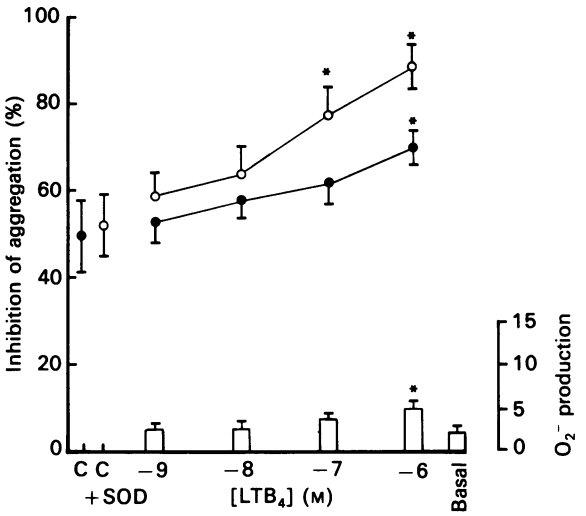

Fig. 3. Effect of LTB_4 on the inhibition of platelet aggregation and on O_2^- generation by PMN

'C' and ● represent the anti-aggregating actions of 3×10^5 PMN, whereas 'C+SOD' and ○ represent that of 1×10^5 PMN in the presence of SOD (60 units·ml^{-1}). Basal and LTB_4-stimulated O_2^- generation is shown by the histogram. * Denotes significant difference from the corresponding control ($n = 4$).

DISCUSSION

Rat peritoneal PMN inhibited platelet aggregation in a cell-concentration-dependent manner. This anti-aggregating activity was potentiated by SOD and by a selective cyclic GMP phosphodiesterase inhibitor, M&B 22948, and was inhibited by haemoglobin and by treatment of the cells with glutaraldehyde. This indicates that viable PMN release NO, which inhibits platelet aggregation via elevation of cyclic GMP [13].

The anti-aggregating activity of PMN was significantly reduced by L-NMMA, but not by D-NMMA, and this effect was reversed by L-arginine. This shows that NO is formed from L-arginine in these cells and that this occurs via an enzymic mechanism similar to that in the vascular endothelium [5,15] and the macrophage [6,16]. The substrate and inhibitor profile for NO synthesis in PMN is similar to that in macrophages [6], but differs somewhat from that in endothelial cells [5] and endothelial-cell homogenates [15], since L-canavanine is an inhibitor in macrophages [17] and in PMN, but not in vascular endothelial cells.

The long period of preincubation required for L-NMMA to inhibit NO formation probably reflects slow uptake into the cells, since similar long periods of preincubation were also required for L-canavanine. The failure of L-NMMA to cause complete inhibition of NO synthesis may indicate the existence of an enzyme within the cell which is inaccessible to L-NMMA. Alternatively, it may reflect another mediator or pathway of NO synthesis. This, however, is unlikely, since haemoglobin abolished the anti-aggregating effect, and a greater inhibition was observed if L-NMMA was included during the preparation of the PMN (results not shown).

Stimulation of PMN with low concentrations of FMLP enhanced the anti-aggregating effect. In contrast, higher concentrations of FMLP not only reversed this enhancement, but also reduced the anti-aggregating activity to below the control level. Since NO is inactivated by O_2^- [4] and these concentrations of FMLP induce its release from PMN, this inhibition is probably due to inactivation of NO by O_2^-, for SOD prevented this effect.

LTB_4 also enhanced the anti-aggregating activity of PMN. However, a reversal of this effect was not observed with high concentrations of LTB_4, and this agonist only released small amounts of O_2^- at these concentrations. This indicates not only that LTB_4, unlike FMLP, is a weak stimulator of NO generation, but also that it does not release sufficient O_2^- to overcome the anti-aggregating effect of NO. Interestingly, L-arginine, like SOD, potentiated the anti-aggregating activity induced by low concentrations of FMLP and inhibited the reversal of this action observed with high concentrations of this agonist. This shows that the same biological effect can be achieved either by scavenging O_2^- (with SOD) or by increasing the levels of NO (with L-arginine).

The precise biological role of NO in PMN remains to be elucidated in detail. NO may, by inactivating O_2^-, control the levels of this anion. If this is the case, then at lower degrees of stimulation such as may occur during margination or emigration, the balance in favour of NO may allow the PMN to leave the vascular system without damaging the endothelium. At higher degrees of stimulation, increased release of O_2^- may overcome this control mechanism, so that the PMN exert their microbiocidal action. In addition, NO may activate the soluble guanylate cyclase to alter PMN function or it may induce metabolic changes that favour their microbiocidal action, as in the macrophage [14]. Whether some of these functions are dependent on the transfer of PMN-derived NO to other cells, such as the vascular endothelium or the platelets, and whether the synthesis of O_2^- and NO are linked in some way [18], require elucidation.

The L-arginine/NO biosynthetic pathway is widely distributed and underlies a variety of functions which range from vasodilatation in the vascular system to cytotoxicity in the macrophage [7]. Since it is likely that this is a system of isoenzymes, inhibitors with selectivity towards one or another cell type will help clarify the role of NO in the different cells *in vivo* as well as the origin of the excess NO_3^- excreted by the whole animal [19]. Unlike L-NMMA, which is a general inhibitor of the pathway, L-canavanine shows selectivity towards the enzyme in the macrophage [17] and the PMN, and is therefore suitable for investigating the role of NO derived from phagocytes.

REFERENCES

1. Rimele, T. J., Sturm, R. J., Adams, L. M., Henry, D. E., Heaslip, R. J., Weichman, B. M. & Grimes, D. (1988) J. Pharmacol. Exp. Ther. **245**, 102–111
2. Cynk, E., Kondo, K., Salvemini, D., Sneddon, J. M. & Vane, J. R. (1988) J. Physiol. (London) **407**, 28*P*
3. Snyderman, R. & Pike, M. C. (1984) Annu. Rev. Immunol. **2**, 257–281
4. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) Nature (London) **327**, 524–526
5. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) Nature (London) **333**, 664–666
6. Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) Science **235**, 473–476
7. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1989) Biochem. Pharmacol. **38**, 1709–1715

8. Radomski, M. W. & Moncada, S. (1983) Thromb. Res. **30**, 383–389
9. Palmer, R. M. J., Stepney, R. J., Higgs, G. A. & Eakins, K. E. (1980) Prostaglandins **20**, 411–418
10. Rajkovic, I. A. & Williams, R. (1985) J. Immunol. Methods **78**, 35–47
11. Paterson, R. A., Eagles, P. A. M., Young, D. A. B. & Beddell, C. R. (1976) Int. J. Biochem. **7**, 117–118
12. Patthy, A., Bajusz, S. & Patthy, L. (1977) Acta Biochim. Biophys. Acad. Sci. Hung. **12**, 191–196
13. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) Br. J. Pharmacol **92**, 181–187
14. Drapier, J.-C. & Hibbs, J. B. (1988) J. Immunol. **140**, 2829–2838
15. Palmer, R. M. J. & Moncada, S. (1989) Biochem. Biophys. Res. Commun. **158**, 348–352
16. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) Biochemistry **27**, 8706–8711
17. Iyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6369–6373
18. Kamoun, P. P., Schneider, E. & Dy, M. (1988) FEBS Lett. **226**, 285–286
19. Green, L. C., Tannenbaum, S. R. & Goldman, P. (1981) Science **212**, 56–58

Received 13 March 1989/13 April 1989; accepted 28 April 1989